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13. ABSTRACT (Maximum 200 Words)
Nkx3.1 is a prostatic-specific tumor suppressor whose loss-of-function represents a critical step in prostate cancer initiation. However, the molecular basis is still largely unknown. We have been utilizing microarray analysis to pursue the gene expression profiling of prostatic lesions in the Nkx3.1 mutant mouse model relative to normal prostate epithelium. Our findings suggest that Nkx3.1 loss-of-function leads to a deregulated secretory function of prostate which representing a defect differentiation of prostate epithelium, thus may contribute to the increased susceptible to carcinogenesis. Moreover, Nkx3.1 mutant prostates are deficient for anti-oxidative protection as a consequence of aging, which underlies its role in cancer predisposition. In an effort to explore the role of Nkx3.1 in advanced stages of prostate cancer progression, gene expression profiling has been performed using Nkx3.1; Pten double mutant and Nkx3.1;Pten;$p27^{kip1}$ triple mutant prostatic lesions. Our preliminary data have shown that Nkx3.1 loss-of-function cooperates with heterozygosity of $p27^{kip1}$ in promotion of prostate carcinogenesis through up-regulation of cyclin D1. Our findings provide insight into the roles of Nkx3.1, by itself or in cooperation with other broad-spectrum tumor suppressors, in prostate carcinogenesis.

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I. Training accomplishments

During the funding period of March 2003 through February 2004, I have received extensive training under the guidance of the supervisor, Dr. Cory Abate-Shen, as I have made considerable progress toward identifying *Nkx3.1*-responsive genes involved in prostate carcinogenesis. With this guidance and training, I have established and optimized the methodologies needed for this study, including laser capture microdissection, RNA isolation and amplification, Affymetrix microarray and data analysis, real-time RT-PCR, *in situ* hybridization and immunohistochemistry. With the help of Whitney Banach-Petrosky, a senior technician of our laboratory, I have also set up a tissue/cell recombination assay for functional study of candidate target genes of *Nkx3.1* during prostate differentiation and/or carcinogenesis.

In addition to the technical support, intellectually, my study has also benefited from the guidance and assistance of Dr. Abate-Shen and other laboratory members. On a weekly basis, I discuss the research project in detail with Dr. Abate-Shen; and on a bi-weekly basis, I present the data at the laboratory meetings. These discussions of the progress and pitfalls of my research project have promoted critical evaluation of the data on a consistent basis. Moreover, many research investigators at CABM and Cancer Institute of New Jersey have provided valuable advices on my research project in many aspects. These include our collaborators, Dr. Michael Shen, who has extensive expertise in prostate biology; and Drs. Yong Lin and Weichung J. Shih, who have provided considerable assistance in microarray data analysis.

Finally, I had the opportunities to attend several symposiums/retreats at CABM, Cancer Institute, and the UMDNJ-Rutger's life science community during last funding period. I will also be presenting two abstracts (one on myself and one as a coauthor) at the 95th annual meeting of American Association for Cancer Research in March 2004.

II. Research accomplishments

A. Introduction

Our **hypothesis** is that *Nkx3.1* homeobox gene functions as a transcription factor to regulate gene expression during normal prostate growth and differentiation, and that loss of *Nkx3.1* leads to the aberrant expression of target genes that ultimately contribute to prostate carcinogenesis. Therefore, identification of *Nkx3.1* target genes will provide insight into the molecular pathways associated with prostate carcinoma.

We have demonstrated that the *Nkx3.1* mutant mice display histopathological features of prostatic intraepithelial neoplasia (PIN), the presumed precursor of human prostate cancer, which increase in severity with age (1, 2). Furthermore, prostatic-specific loss-of-function of *Nkx3.1* cooperates with loss-of-function of broad-spectrum tumor suppressors such as *Pten* and/or $p27^{kip1}$ in cancer progression (3, 4).

Using Affymetrix GeneChip expression profiling, as validated by real-time RT-PCR and immunohistochemistry, we have now found that loss-of-function of *Nkx3.1* in mice leads to a deregulated secretory function in prostate, which represents a less differentiated secretory tissue and may contribute to increased susceptibility to prostate carcinogenesis. Furthermore, our findings suggest *Nkx3.1* loss-of-function leads to accumulation of oxidative damage in prostate as a consequence of aging, which underlies its role in cancer predisposition.

To explore *Nkx3.1*-responsive genes during more advanced stages of prostate cancer progression, and to verify the synergistic activity of *Nkx3.1*, *Pten* and/or $p27^{kip1}$, *Nkx3.1;Pten* and *Nkx3.1;Pten;p27* compound mutant prostates were analyzed microarray analysis accompanied with the laser capture microdissection (LCM) technique. Our findings suggest that the molecular mechanisms that mediate this cooperativity include the activation of *cyclin D1*, a key modulator of cell proliferation.

Taken together, our findings underscore the significance of *Nkx3.1* loss-of-function, by itself, or cooperating with other putative tumor suppressors such as *Pten* and $p27^{kip1}$, in prostate cancer initiation and progression.

B. Body Below we list our goals for this year (from the statement of work) and a description of the status.

Task 1. Isolation of *Nkx3.1*-responsive genes by microarray analysis.

This goal has been successfully implemented. We compared mRNA from prostates (anterior and dorsolateral lobes) of *Nkx3.1* homozygous mutant and wild-type mice at 15-month-old of age. Microarray analysis was done in triplicate using mRNA samples comprised of 3 mutant or 3 wild-type prostates; this '3×3 protocol' minimizes artifacts that arise from individual mRNA samples. Data analyses were done using ANOVA method followed by multiplicity adjustment using Holm or B&H procedure. As visualized by two-way hierarchical clustering, a total of 638 genes were differentially expressed following *Nkx3.1* loss-of-function. Among them are deregulated expression of 4 genes encoding seminal vesicle secretory proteins and prostatic specific probasin (Figure 1A). Moreover, of particular interest was aberrant levels of several anti-oxidation enzyme in *Nkx3.1* mutant prostates, suggesting loss-of-function of *Nkx3.1* predisposes to prostate cancer through increased oxidative damage (Figure 2A). Validations of microarray findings have been done as described in progress of Task 4.

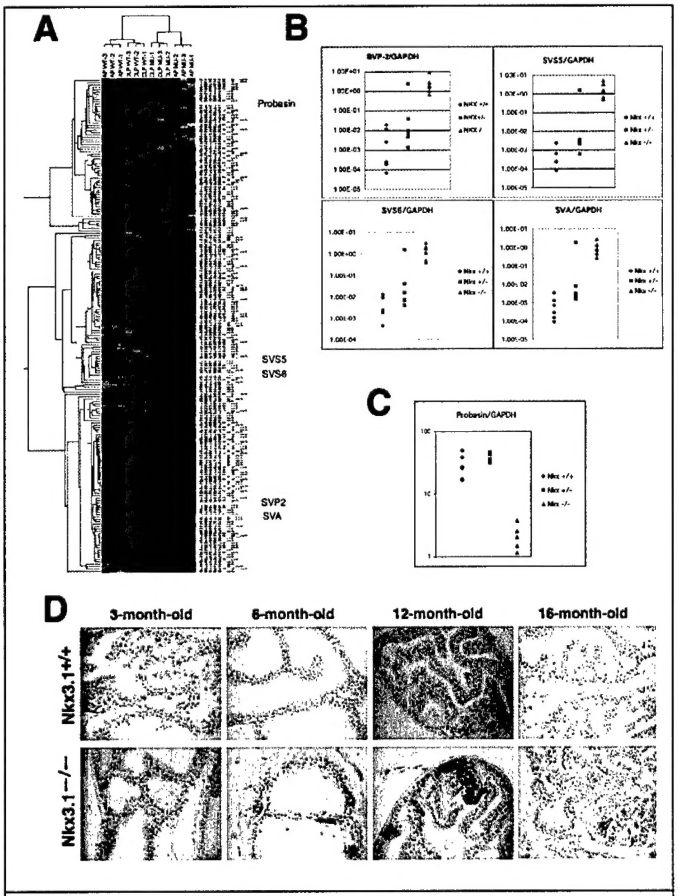

Figure 1. Deregulated secretory function of *Nkx3.1* mutant prostates. A. Gene expression profiling comparing *Nkx3.1* wild-type and mutant prostates (dosalateral and anterior lobes). Among a total of 638 deregulated genes are genes encoding seminal vesicle or prostate secretory proteins, including *SVP2*, *SVS5&6*, *SVA* and *probasin*. **B.** Real-time RT-PCR analyses of mRNA levels of seminal vesicle secretory proteins. **C.** Real-time RT-PCR analysis of probasin. **D.** Immunohistochemical analysis of SVP2 in anterior prostates from *Nkx3.1* wild-type or mutant mice of different ages.

We have also performed gene expression profiling of LCM samples using 4 prostatic lesions (hyperplasia & low-grade PIN) of *Nkx3.1* mutant mice and 4 normal prostatic epitheliums of age-matched wild-type mice. Data analysis is now carrying out.

However, the comparison of prostatic lesions *vs.* adjacent normal prostatic epithelium of $Nkx3.1^{-/-}$ mice is technically difficult since the heterogeneity and multifocality of the lesions in *Nkx3.1* mutant prostate made them hard to be captured from cryosections. Instead, comparison of lesions and normal prostatic epitheliums was performed using different samples.

Task 2. Isolation of target genes for synergistic activity of *Nkx3.1* and *Pten* loss-of-function by microarray analysis.

Microarray experiment has been performed using LCM samples to compare the high-grade or low-grade PIN lesions of *Nkx3.1;Pten* compound mutants (n=9) *vs.* normal prostate epitheliums of age-matched $Nkx3.1^{+/+}$ mice (n=4). However, I have not further pursued the comparison of PIN lesions *vs.* adjacent normal epitheliums *Nkx3.1;Pten* compound mutants because of the similar aforementioned technical impractibility.

Task 3. Exploration of *Nkx3.1* regulated genes in the development of PIN to prostate cancer.

We proposed to explore *Nkx3.1*-responsive genes in advanced stages of prostate carcinogenesis using a tissue recombination approach. However, our recent studies have shown that *Nkx3.1;Pten* and *Nkx3.1;Pten;p27^{kip1}* compound mutants recapitulate various stages of prostate carcinogenesis from low-grade PIN to high-grade PIN, to invasive adenocarcinoma, and metastases to the distant tissues (3-5). Therefore, we have not pursued the tissue recombination approach. Instead, by using the double (*Nkx3.1;Pten*) and triple (*Nkx3.1;Pten;p27^{kip1}*) compound mutants, we extended our proposed task 3 not only to explore *Nkx3.1*-responsive genes, but also to explore the cooperativity of prostate-specific tumor suppressor *Nkx3.1* and broad-spectrum tumor suppressors such as *Pten* and *p27^{kip1}* during multi-stages of prostate cancer progression.

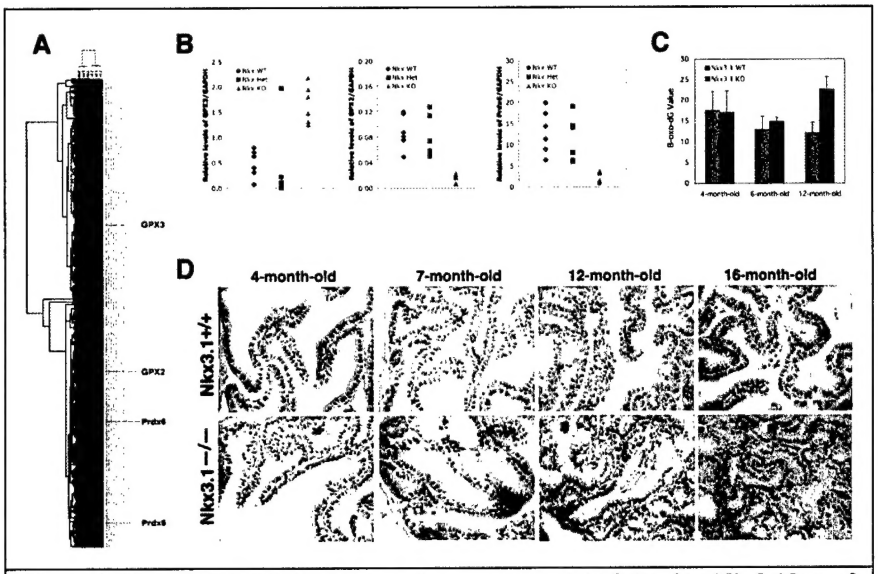

Figure 2. Increased oxidative damage in mouse prostate following *Nkx3.1* loss-of-function. A. Gene expression profiling comparing *Nkx3.1* wild-type and mutant anterior prostates with highlights of genes encoding anti-oxidation enzymes, including *GPX2&3* and *Prdx6*. **B.** Real-time RT-PCR analyses of *GPX3*, *GPX2* and *Prdx6* levels in anterior prostates from *Nkx3.1* wild-type, heterozygous and homozygous mutant mice. **C.** Increased 8-oxo-dG levels in *Nkx3.1* mutant anterior prostates as a consequence of aging. **D.** Immunohistochemical analyses to show elevated iNOS levels in anterior prostates from aged *Nkx3.1* mutant mice.

For this purpose, we have performed gene expression profiling using LCM samples of 9 double (*Nkx3.1;Pten*) mutants and 7 triple (*Nkx3.1;Pten;p27^{kip1}*) mutants, which representing the progression of prostate cancer from PIN lesions to invasive adenocarcinoma. Our preliminary microarray data analyses of triple mutants have revealed that *Nkx3.1* loss-of-function cooperates with heterozygosity of *p27^{kip1}* in promotion of prostate carcinogenesis through up-regulation of *cyclin D1* (Figure 3).

Task 4. Validation of *Nkx3.1*-responsive genes.

As mentioned in summary of Task 1, microarray analyses of *Nkx3.1* mutant and wild-type prostates have revealed a robust increase in expression of 4 genes encoding secretory proteins that are normally expressed by the seminal vesicle: (i) seminal vesicle secretory protein 2 (*SVP2*; ~500 fold); (ii) seminal vesicle secretion 5 (*SVS5*; ~210 fold); (iii) seminal vesicle secretion 6 (*SVS6*; ~38 fold); and (iv) seminal vesicle autoantigen (*SVA*; ~20 fold). Meanwhile, genes encoding prostatic secretory proteins, such as *probasin*, were reduced (by ~3 fold) in *Nkx3.1* mutant prostate (Figure 1A). These findings have been further confirmed by Real-time RT-PCR (Figure 1B&C) and immunohistochemistry study (Figure 1D). We thus propose that *Nkx3.1* is required for prostatic epithelial specification; in its absence the prostatic epithelial of *Nkx3.1* mutants represents defects in differentiation which may contribute to the increased susceptible to carcinogensis. To test this hypothesis, we have developed two immortalized cell lines from embryonic urogenital epithelium (UGE) or seminal vesicle epithelium (SVE) for functional study of *Nkx3.1* using a tissue/cell recombination approach. We are using the *Nkx3.1* mutants and UGE/SVE cells as models to study the consequences of aberrant differentiation for cancer susceptibility in prostate.

Furthermore, gene expression profiling, as validated by real-time RT-PCR, have also revealed aberrant expression of several anti-oxidation enzymes following *Nkx3.1* loss-of-function, including glutathione peroxidase 2&3 (*GPX2, GPX3*), and peroxiredoxin 6 (*Prdx6*) (Figure 2A&B). Further examine of oxidative DNA damage by analyzing the levels of 8-oxo-2'-deoxyguanosine (8-oxo-dG) in genomic DNA has demonstrated increased 8-oxo-dG levels in the *Nkx3.1* mutant prostates compared with age-matched controls (Figure 2C). We have also observed a marked enhancement in induced nitric oxide synthases (iNOS) immuno-reactivity in *Nkx3.1* mutant prostate, compared to the age-matched wild-type prostate (Figure 2D), suggesting that the mutant may have elevated levels of nitric oxide, a radical, nitrosating agent, and indirect mutagenic oxidant. Taken together, loss-of-function of *Nkx3.1* may lead to accumulation of oxidative damage in prostate as a consequence of aging, which underlies its role in cancer predisposition.

C. Key research accomplishments

- Gene expression profiling of *Nkx3.1* wild-type and mutant prostates reveal a total of 638 genes are differentially expressed following *Nkx3.1* loss-of-function;
- Deregulated secretory function in *Nkx3.1* mutant prostate reflects a defective differentiation of prostate which may contribute to the increased susceptible to carcinogensis;
- Loss-of-function of *Nkx3.1* leads to accumulation of oxidative damage in prostate
- *Nkx3.1* cooperates with heterozygosity of $p27^{kip1}$ in promotion of prostate carcinogenesis through up-regulation of *cyclin D1.*
- A large-scale microarray analysis has been done, including 4 wild-type normal prostatic epitheliums, 4 hyperplastic/low-grade PIN lesions from $Nkx3.1^{-/-}$ prostates, 9 PIN lesions or adenocarcinomas from *Nkx3.1;Pten* double mutant prostates, and 7 PIN lesions or adenocarcinomas from *Nkx3.1;Pten;*$p27^{kip1}$ triple mutant prostate. Data analysis is carrying out in collaboration with Drs. Yong Lin and Weichung J Shih at Cancer Institute of New Jersey.

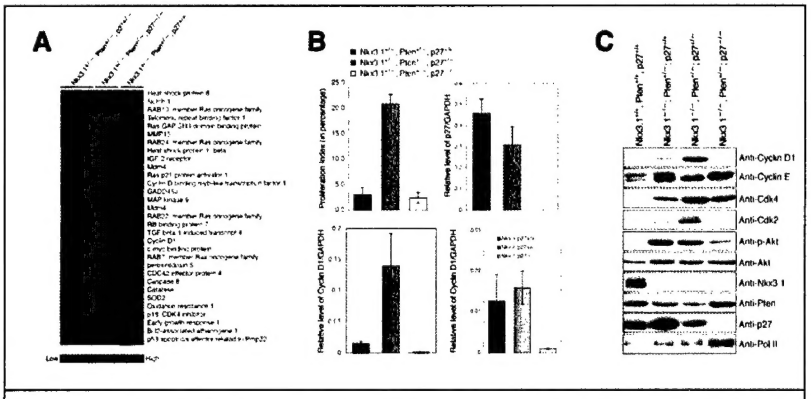

Figure 3. Up-regulation of cyclin D1 in compound heterozygotes. A. Gene expression profiling comparing compound mutant mice having two, one, or no alleles of $p27^{kip1}$. RNA was obtained by LCM from dorsolateral prostate to isolate prostate PIN or cancer cells. Selected cancer related genes are shown. Among those is cyclin D1. **B.** Expression analyses. Quantitation of the proliferation of prostate epithelial cells in PIN and cancer lesions from compound mutant mice (*upper left*); Real-time RT-PCR analyses of $p27^{kip1}$ (*upper right)* and *cyclin D1* (*bottom left*) in LCM isolated prostatic PIN or cancer cells from compound mutant mice; Real-time RT-PCR analyses of *cyclin D1* in LCM isolated prostatic epithelium from *Nkx3.1;*$p27^{kip1}$ double compound mice (*bottom right*). **C.** Western blot analyses. Whole cell extracts were made from anterior prostate of mice of the indicated genotypes. Pol II detects the large subunit of RNA polymerase II is an internal control for protein loading.

D. Reportably outcomes

- Abstracts

1. **Ouyang, X.S.**, DeWeese, T.L., Nelson, W.G., Abate-Shen, C. (2004) Loss of function of Nkx3.1 predisposes to prostate cancer through increased oxidative damage as a consequence of aging. *Pro. Am. Assoc. Cancer Res.* 45: No. 770.
2. Gao, H., Banach-Petrosky, W.A., **Ouyang, X.S.**, Sun, X., Kim, M., Lee, H., Lin, Y., Shih, W.J., Borowsky, A.D., Cardiff, R.D., Shen, M.M., Abate-Shen, C. (2004) Heterozygosity of p27kip1 promotes prostate carcinogenesis through up-regulation of cyclin D1. *Pro. Am. Assoc. Cancer Res.* 45: No. 5560.

- Manuscripts

1. Gao, H., **Ouyang, X.S.**, Banach-Petrosky, W.A., Sun, X., Kim, M., Lee, H., Lin, Y., Shih, W.J., Borowsky, A.D., Cardiff, R.D., Shen, M.M., Abate-Shen, C. Heterozygosity of p27kip1 promotes prostate carcinogenesis through up-regulation of cyclin D1. (submitted)
2. **Ouyang, X.S.**, DeWeese, T.L., Nelson, W.G., Abate-Shen, C. Loss of function of Nkx3.1 predisposes to prostate cancer through increased oxidative damage as a consequence of aging. (In perparation)
3. **Ouyang, X.**S., Banach-Petrosky, W.A., Shen, M.M., Abate-Shen, C. The role of *Nkx3.1* in organ specification of the prostate. (In preparation)

E. Conclusions

Several lines of evidence have implicated that *Nkx3.1* is a prostatic-specific tumor suppressor whose loss-of-function represents a critical step in prostate cancer initiation. However, the molecular basis is still largely unknown. Using the *Nkx3.1* mutant mouse model, we have performed a broad exploration using Affymetrix gene expression profiling approach, followed by a validation of several specific genes using real-time RT-PCR and immunohistochemistry. Our findings suggest that *Nkx3.1* is required for prostatic epithelial specificity and its loss-of-function leads to deficient secretory function in prostate, which may contribute to the increased susceptible to carcinogenesis.

Moreover, our findings have revealed that loss-of-function of *Nkx3.1* lead to a loss of anti-oxidative protection in prostate as a consequence of aging, which underlies its role in cancer predisposition.

To explore the role of *Nkx3.1* in advanced stages of prostate cancer progression, gene expression profiling has been performed using *Nkx3.1;Pten* double mutant and *Nkx3.1;Pten;p27^{kip1}* triple mutant prostatic lesions. Our preliminary data have shown that *Nkx3.1* loss-of-function cooperates with heterozygosity of *p27^{kip1}* in promotion of prostate carcinogenesis through up-regulation of *cyclin D1*. Upon the completion of microarray data analysis, more interesting genes will be followed up for functional studies to define the succession of molecular events that culminate in prostate carcinoma.

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